



UNIVERSITI PUTRA MALAYSIA

**DEVELOPMENT OF A PLANT REGENERATION SYSTEM AND
ANALYSIS OF 101 HEAT SHOCK PROTEIN IN STRAWBERRY cv.
CAMAROSA FOLLOWING GENE BOMBARDMENT**

FATEMEH HADDADI

FP 2009 11

**DEVELOPMENT OF A PLANT REGENERATION SYSTEM AND
ANALYSIS OF 101 HEAT SHOCK PROTEIN IN STRAWBERRY cv.
CAMAROSA FOLLOWING GENE BOMBARDMENT**

By

FATEMEH HADDADI

**Thesis Submitted to the School of Graduate Studies, Universiti
Putra Malaysia, in Fulfilment of the Requirement for the Degree of
Master of Science**

2009



In The Name of Allah
The Most Gracious, the Most Merciful

Specially dedicated to:

My beloved
Hossein

And

My kind parents
Habib and Parvin

Abstract of thesis presented to the Senate of Universiti Putra Malaysia
in fulfillment of the requirement for the degree of Master of Science

**DEVELOPMENT OF A PLANT REGENERATION SYSTEM AND
ANALYSIS OF 101 HEAT SHOCK PROTEIN IN STRAWBERRY cv.
CAMAROSA FOLLOWING GENE BOMBARDMENT**

By

FATEMEH HADDADI

February 2009

Chairperson: Associate Professor Maheran Abdul Aziz, PhD

Faculty : Agriculture

The aims of this study were to develop *in vitro* regeneration system and to confirm the transient expression of HSP101 gene via protein analysis in strawberry cv. Camarosa.

In the *in vitro* study, two types of explants which were shoot tips derived from runner tips and leaves were used. Different types of cytokinins such as BAP, TDZ and zeatin at different concentrations were assessed for shoot induction, while the auxins IBA and NAA also at different concentration were used in the root induction experiment. The experiments were conducted in a Randomized Complete Block Design (RCBD).

In the shoot induction experiment using shoot tips cultured on different concentrations and combinations of TDZ and BAP, MS medium

supplemented with 4 μ M BAP in combination with 2 μ M TDZ was optimum for strawberry shoot proliferation. In the shoot induction experiment from shoot tips using zeatin, the highest percentage of explant producing shoots and number of shoots formed per explant were obtained on MS medium containing 4 μ M zeatin. High frequency of shoot regeneration from strawberry leaves using different concentrations and combinations of BAP and TDZ was achieved on MS medium containing 4 μ M TDZ, without BAP. In the rooting study, MS medium containing 1 μ M NAA, MS medium containing 1 μ M IBA and MS medium without auxins, were most suitable in inducing the highest number of roots per explant, highest percentage of root formation and the longest root, respectively.

Biolistic method of gene transfer has the advantage of allowing a fast and rapid analysis, and was therefore selected for transient expression of HSP101 gene in strawberry via protein analysis. In this study, *in vitro* leaf explants of strawberry were used. Transient gene expression assays of the *AtHSP101* gene showed that this gene can be transiently expressed in strawberry plants. An additional faint protein band of approximately 100 kD was observed on SDS polyacrylamide gel electrophoresis after bombardment of the leaf explant with plasmid, which most probably corresponded to the HSP101 encoded product. In the study on total protein assay using Bradford method, the amount of total protein after bombardment of leaf explant with plasmid containing

HSP101 gene increased in comparison with bombardment without plasmid and with non bombarded explants. This result also confirmed that this gene can be transiently expressed in strawberry plants. Therefore by using the regeneration protocol obtained in this study and HSP101 gene which can be transiently expressed, genetic engineering of strawberry cv. Camarosa for heat tolerance can be achieved.

Abstrak tesis dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan Ijazah Master Sains

**PEMBENTUKAN SISTEM REGENERASI TUMBUHAN DAN
ANALISA PROTEIN KEJUTAN HABA 101 KEATAS STRAWBERI
cv. CAMAROSA SELEPAS PEMBEDILAN GEN**

Oleh

FATEMEH HADDADI

Februari 2009

Pengerusi : Professor Madya Maheran Abdul Aziz, PhD

Fakulti : Pertanian

Tujuan kajian ini adalah untuk membangunkan sistem regenerasi secara *in vitro* dan untuk memastikan pengekspresan transien gen HSP101 melalui analisa protein pada strawberi cv. Camarosa.

Dalam kajian *in vitro*, dua jenis eksplan iaitu tunas hujung daripada hujung 'runner' dan daun digunakan. Jenis sitokinin yang berbeza seperti BAP, TDZ dan zeatin pada kepekatan yang berbeza telah diuji untuk induksi tunas, manakala auksin IBA dan NAA juga pada kepekatan yang berbeza digunakan untuk kajian induksi akar. Eksperimen dilaksanakan dalam Rekabentuk Blok Berawak Penuh (RCBD).

Dalam kajian induksi tunas menggunakan tunas hujung yang dikultur pada kepekatan dan kombinasi TDZ dan BAP yang berbeza, medium MS yang ditambahkan dengan 4 μ M BAP dengan kombinasi 2 μ M TDZ adalah optima untuk penggandaan tunas strawberi. Dalam kajian induksi tunas daripada tunas hujung menggunakan zeatin, peratus eksplan yang mengeluarkan tunas dan bilangan tunas terbentuk per eksplan yang paling tinggi diperoleh pada medium MS yang mengandungi 4 μ M zeatin. Frekuensi regenerasi tunas yang tinggi daripada daun strawberi diperoleh pada medium MS yang mengandungi 4 μ M TDZ tanpa BAP. Dalam kajian pengakaran, medium MS yang mengandungi 1 μ M NAA, medium MS yang mengandungi 1 μ M IBA dan medium MS tanpa auksin, masing-masing adalah sangat sesuai untuk induksi bilangan akar per eksplan paling tinggi, peratusan pembentukan akar paling tinggi dan akar yang terpanjang.

Pemindahan gen melalui kaedah biolistik mempunyai kelebihan ke arah analisis yang cepat dan segera, maka dipilih untuk pengekspresan transien gen HSP101 di dalam strawberi melalui analisis protein. Dalam kajian ini, eksplan daun *in vitro* strawberi telah digunakan. Esei pengekspresan gen transien bagi gen *AtHSP 101* menunjukkan bahawa gen ini dapat diekspresikan dalam tumbuhan strawberi. Tambahan jalur protein saiz 100 kD yang samar dilihat pada elektroforesis gel poliakrilamid selepas pembedilan eksplan daun, yang

berkemungkinan besar produk pengkodan HSP101. Dalam kajian esei protein total menggunakan kaedah Bradford, kandungan protein total selepas pembedilan eksplan daun dengan plasmid yang mengandungi gen HSP101 meningkat berbanding dengan pembedilan tanpa plasmid dan dengan eksplan tanpa pembedilan. Hasil ini juga memastikan bahawa gen ini dapat diekspresikan secara transien di dalam tumbuhan strawberi. Oleh itu, dengan menggunakan protokol regenerasi yang telah diperoleh dan gen HSP101 yang boleh diekspresi, kejuruteraan genetik strawberi cv. Camarosa untuk toleransi terhadap haba boleh dicapai.

ACKNOWLEDGEMENTS

My deepest gratitude to God for his indescribable help, for all thou dost supply, for comfort in despair, and for giving me the grace to complete another step of my life, and the best regards from God to the last prophet, Mohammad and his family.

I would like to express my special thanks to the Chairman of my Supervisory Committee, Associate Professor Dr. Maheran Abdul Aziz of the Department of Agriculture Technology, Faculty of Agriculture, for her useful advices, for her friendship and for giving me the opportunity and freedom to do my favourite project.

I owe my thanks to the member of my Supervisory Committee, Professor Dr. Ghizan Saleh, Dean of the Faculty of Agriculture, for giving me valuable advices and useful discussions during my research.

I am also thankful to Mr. Azmi Abdul Rashid for his guidance and constant encouragement during my research and also to Associate Professor Datin Dr. Siti Nor Akmar Abdullah for allowing me to carry out my research in her laboratory.

I appreciate all of my lab mates and my friend Arash Rafat, Mohamad Bagher Javadi, Hossein Torabi Sirchi, Vahid Omidvar, Pedram

Kashiani, Ramtin Ravanfar, Esmaeil Shahsavari, Syaiful, Suleiman, Armiyanti, Rozila and especially my best friends Azadeh Niknejad, Motahareh Nobakht and Naghmeh Nejat who made my life enjoyable in Malaysia. I am also grateful to Arash Rafat for kindly providing the pCAMHSP vector.

Lastly I would like to express my special thanks and deepest gratitude to my loving husband Hossein, my beloved parents Mr. Habib and Mrs. Parvin, and my brothers Farshad and Farzad for their patience, kindness, and grateful support throughout my life and my study.

I certify that an Examination Committee has met on November 2008 to conduct the final examination of Fatemeh Haddadi on her Master of Science thesis entitled “Development of a Plant Regeneration System and Analysis of 101 Heat Shock Protein in Strawberry cv. Camarosa Following Gene Bombardment” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the student be awarded the Master Science.

Members of the Examination Committee were as follows:

Datin Siti Nor Akmar Abdullah, PhD

Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairperson)

Mihdzar Abdul Kadir, PhD

Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal examiner)

Faridah Qamaruz Zaman, PhD

Senior Lecturer
Faculty of Science
Universiti Putra Malaysia
(Internal examiner)

Normah Mohd Noor, PhD

Professor
Institute of System and Technology
Universiti of kebangsaan Malaysia
Malaysia
(External Examiner)

BUJANG KIM HUAT, PhD

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 29 May 2009

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of Supervisory Committee are as follows:

Maheran Abdul Aziz, PhD

Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Ghizan Saleh, PhD

Professor/Dean
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

HASANAH MOHD. GHAZALI, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 8 June 2009

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged .I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or any other institution.

FATEMEH HADDADI

NAME OF STUDENT

Date:

TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	vi
ACKNOWLEDGEMENTS	ix
APPROVAL	xi
DECLARATION	xiii
LIST OF TABLES	xvii
LIST OF FIGURES	xviii
LIST OF ABBREVIATIONS	xxi
 CHAPTER	
	1
1 INTRODUCTION	4
2 LITERATURE REVIEW	
2.1 History of Strawberry Cultivation	4
2.2 Botany and Taxonomy of Strawberry	5
2.3 Biology of Strawberry	6
2.4 Camarosa Cultivar	8
2.5 Plant Growth Regulator	9
2.6 Strawberry <i>In Vitro</i> Culture	11
2.7 Transformation Techniques	15
2.7.1 Transient Assay	16
2.7.2 Heat Shock Proteins	18
2.7.3 Heat Shock Protein 101	19
2.7.4 High Temperature Stress	20
2.8 Bradford Protein Assay	21
3 STRAWBERRY <i>IN VITRO</i> CULTURE: SHOOT REGENERATION AND ROOT FORMATION	23
3.1 Introduction	23
3.2 Objectives	24
3.3 Materials and Methods	25
3.3.1 Location of Study	25
3.3.2 Stock Plant Materials	26
3.3.3 Runner Tips Surface Sterilization Procedure	27
3.3.4 Basic Medium	28
3.3.5 Culture Condition	28
3.4 Effects of Different Concentrations of BAP in Combination with TDZ on Shoot Induction from Shoot Tips Derived from the <i>In Vitro</i> Stock Plant	28
3.4.1 Plant Material	28
3.4.2 Treatments	28

3.4.3	Measurements Taken	30
3.5	Effects of Different Concentrations of Zeatin on Shoot Induction from Shoot Tips Derived from the <i>In Vitro</i> Stock Plant	30
3.5.1	Plant Material	30
3.5.2	Treatments	30
3.5.3	Measurements Taken	31
3.6	Effects of Different Concentrations of BAP in Combination with TDZ on Shoot Regeneration from Strawberry Leaves	31
3.6.1	Plant Material	31
3.6.2	Treatments	32
3.6.3	Measurements Taken	33
3.7	Effects of Different Types and Concentrations of Auxins, NAA and IBA, on <i>In Vitro</i> Rooting of Strawberry Shoots	33
3.7.1	Plant Material	33
3.7.2	Treatments	33
3.7.3	Measurements Taken	34
3.8	Statistical Analysis	34
3.9	Results	35
3.9.1	Effects of Different Concentrations of BAP in Combination with TDZ on Shoot Induction from Shoot Tips Derived from the <i>In Vitro</i> Stock Plant	35
3.9.2	Effects of Different Concentrations of Zeatin on Shoot Induction from Shoot Tips Derived from the <i>In Vitro</i> Stock Plant	39
3.9.3	Effects of Different Concentrations of BAP in Combination with TDZ on Shoot Regeneration from Strawberry Leaves	43
3.9.4	Effects of Different Types and Concentrations of Auxins, NAA and IBA, on <i>In Vitro</i> Rooting of Strawberry Shoots	46
3.10	Discussion	52
3.10.1	Effects of Different Concentrations of BAP in Combination with TDZ on Shoot Induction from Shoot Tips Derived from the <i>In Vitro</i> Stock Plant	52
3.10.2	Effects of Different Concentrations of Zeatin on Shoot Induction from Shoot Tips Derived from the <i>In Vitro</i> Stock Plant	55
3.10.3	Effects of Different Concentrations of BAP in Combination with TDZ on Shoot Regeneration from Strawberry Leaves	56
3.10.4	Effects of Different Types and Concentrations of Auxins, NAA and IBA, on <i>In Vitro</i> Rooting of Strawberry shoots	59

4	HSP101 GENE BOMBARDMENT AND PROTEIN ANALYSIS	62
4.1	Introduction	62
4.2	Objectives	64
4.3	Materials and Methods	65
4.3.1	Location of Study	65
4.3.2	Pre-Bombardment	65
4.3.3	Plasmid Material	65
4.3.4	Plasmid Preparation	66
4.3.5	Chemical Solution Preparation	68
4.3.6	Medium Preparation	68
4.3.7	Explant Material	68
4.3.8	Bombardment Procedures	69
4.3.9	Preparation of Particle Gun	69
4.3.10	Preparation of Microparticles	69
4.3.11	Particle Coating Protocol	70
4.3.12	Bombardment Parameters	71
4.3.13	Culture Condition	71
4.3.14	Treatments	71
4.4	Protein Analysis	72
4.4.1	Explant Material	72
4.4.2	Protein Extraction	73
4.4.3	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)	73
4.4.4	Staining and Restaining of Sodium Dodecyl Sulfate Polyacrylamide Gel	74
4.4.5	Bradford Assay	74
4.4.5.1	Experimental Design and Statistical Analysis	75
4.5	Results	76
4.5.1	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)	76
4.5.2	Bradford Assay	77
4.6	Discussion	79
5	GENERAL DISCUSSION AND COCLUSION	84
5.1	Strawberry <i>In Vitro</i> Culture	84
5.2	HSP101 Gene Bombardment and Protein Analysis	89
	REFERENCES	91
	APPENDICES	105
	BIODATA OF THE STUDENT	117

LIST OF TABLES

Table		Page
2.1	Nutrition information of strawberry	7
3.1	The combinations of different concentrations of BAP and TDZ on shoot induction from shoot tips derived from the <i>in vitro</i> stock plant of strawberry	29
3.2	Different concentrations of zeatin on shoot induction from shoot tips derived from the <i>in vitro</i> stock plant	31
3.3	Different concentrations of BAP in combination with TDZ for shoot regeneration from strawberry leaves	32
3.4	Different concentrations of IBA and NAA on <i>in vitro</i> rooting of strawberry	34
4.1	Different treatments used for gene bombardment of strawberry leaves with HSP101 gene	72

LIST OF FIGURES

Figure		Page
2.1	The model of action of the molecular chaperone HSP90	19
2.2	Schematic diagram of the mechanism of Bradford Assay	22
3.1	Production of <i>in vitro</i> stock materials from strawberry runners on hormone free MS medium, a: Shoots formed after two weeks of culture (Bar = 0.38 cm); b: Further proliferation of shoots after six weeks of culture (Bar = 0.7 cm)	26
3.2	Protocol for sterilization of strawberry runner tips	27
3.3	Shoots of beginning to proliferate after one week of culture on MS medium containing 4 μ M BAP and 2 μ M TDZ (B4T2), (Bar = 0.5 cm)	37
3.4	Shoot formation on MS medium containing 4 μ M BAP and 2 μ M TDZ after 12 weeks of culture (B4T2), arrow shows fasciated shoot, (Bars = 1 cm)	37
3.5	Effect of different concentrations of BAP in combination with TDZ on number of shoot induced from shoot tips derived from the <i>in vitro</i> stock plant after 12 weeks of culture	38
3.6	Effect of different concentrations of BAP in combination with TDZ on percentage of explant producing shoots from shoot tips derived from the <i>in vitro</i> stock plant after 12 weeks of culture	38
3.7	a: Poor shoot formation after eight weeks of culture on MS medium containing 27 μ M BAP and 4 μ M TDZ (B27T4) (Bar = 1 cm); b: Shoot elongating normally after subculturing from MS medium containing 4 μ M BAP and 2 μ M TDZ (B4T2) to hormone free MS medium (Bar = 0.6 cm)	39
3.8	Shoots obtained on MS supplemented with 4 μ M zeatin, a: After three weeks of culture (Bar = 0.75 cm); b: After three subcultures (Bar = 0.77 cm)	41

3.9	Effect of different concentrations of zeatin on number of shoot induced from shoot tips derived from the <i>in vitro</i> stock plant after 12 weeks of culture	41
3.10	Effect of different concentrations of zeatin on percentage of explant producing shoots from shoot tips derived from the <i>in vitro</i> stock plant after 12 weeks of culture	42
3.11	Root formation along with shoot proliferation on MS medium containing 4 μ M zeatin after the first subculture (Bar = 0.7 cm)	42
3.12	Effect of different concentrations of BAP in combination with TDZ on number of shoot produced from strawberry leaves after four weeks of culture	44
3.13	Shoot regeneration after four weeks of culture on MS medium, a: With 4 μ M TDZ without BAP (B0T4) (Bar = 1 cm); b: Without hormone (B0T0) (Bar = 0.8 cm); c: With 4 μ M BAP without TDZ (B4T0) (Bar = 1 cm)	45
3.14	Effect of different concentrations of BAP in combination with TDZ on percentage of explant producing shoots from strawberry leaves after four weeks of culture	46
3.15	Root formation on hormone free MS medium, (Bar = 1 cm)	48
3.16	Effect of different concentrations of IBA and NAA on mean number of roots produced per explant after four weeks of culture	49
3.17	Effect of different concentrations of IBA and NAA on percentage of rooting after four weeks of culture	49
3.18	Effect of different concentrations of IBA and NAA on mean length of root (cm) after four weeks of culture	50
3.19	Callus formation occurring after three weeks of culture on, a: MS medium with 3 μ M NAA, b: MS medium with 3 μ M IBA (Bars = 1 cm)	50
3.20	Callus formation after four weeks of culture on, a: MS medium with 5 μ M IBA with root formation, b: MS medium with 5 μ M NAA without root formation, Yellow	51

arrow: root; White arrows: callus (Bars = 1 cm)

3.21	a: Comparison of root formation, 1: High number of roots formed in MS medium with 1 μ M NAA, 2: High length of root formed in hormone free MS medium; b: Root formation in medium with 5 μ M IBA, roots are thick, (white arrows), (Bar = 1 cm)	51
4.1	a: Plating of <i>Agrobacterium Tumefaciens</i> C58 on LB agar medium with 50 μ g/ml kanamycin; b: <i>Agrobacterium Tumefaciens</i> C58 on LB broth containing 50 μ g/ml kanamycin	67
4.2	Gel electrophoresis of pCAMHSP vector used in the study where; L: Ladder; 1 and 2: pCAMHSP vector (~12.5 Kb)	67
4.3	Analysis of protein profile from bombarded and non bombardment strawberry leaves on 10 % SDS polyacrylamide gel electrophoresis. Arrows show identified band observed after gene bombardment with 10 and 20 μ l HSP101 plasmid	77
4.4	Effect of HSP101 gene expression on total protein of strawberry leaves (determined using Bradford assay)	78

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
<i>AtHSP101</i>	<i>Arabidopsis thaliana</i> heat shock protein 101
BA	N6-benzyladenine
BAP	6-benzylaminopurine
bp	base pairs
BSAA	3-benzo[b] selenienyl acetic acid
CaMV	cauliflower mosaic virus
CaCl ₂	calcium chloride
cDNA	complementary DNA
CoCl ₂ 6H ₂ O	cobalt chloride 6-water
CuSO ₄ 5H ₂ O	cuprum sulfate 5-water
cv.	cultivar
CoCl ₂ 6H ₂ O	cobalt chloride 6-water
DNA	deoxy ribonucleic acid
DNMRT	duncan new multiple range test
EDTA	ethylene diamine tetra acetic acid
<i>E.coli</i>	<i>Escherichia coli</i>
<i>et al.</i>	et alia
FW	fresh weight
GFP	green fluorescent protein
GUS	β-glucuronidase
HCl	hydrochloric acid

HSP	heat shock protein
IAA	indole-3-acetic acid
IBA	Indol-3-Butyricacid
Kb	kilobases
kD	kilodaltons
KH ₂ PO ₄	potassium dihydrogen phosphate
KI	potassium iodide
KNO ₃	potassium nitrate
KOH	potassium hydroxide
LB	Luria Bertani medium
MgSO ₄	manganese sulfate 4-water
μmol m ⁻² s ⁻¹	micromole per square meter per second
MS	Murashige and Skoog
NAA	a-naphthaleneacetic acid
NaCl	sodium hydroxide
Na ₂ MoO ₄ 2H ₂ O	natrium molybdate 2-water
NH ₄ NO ₃	ammonium nitrate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PGR	plant growth regulator
pH	-log (H ⁺)
RCBD	randomized complete block design
RNAase	ribonuclease
rpm	revolutions per minute

SDS	sodium dodecyl sulphate
UV	ultraviolet (light)
v/v	volume to volume
w/v	weight to volume
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	zinc sulfate 7-water
2,4-D	2,4-dichlorophenoxyacetic acid

CHAPTER 1

INTRODUCTION

The strawberry belongs to the *Rosaceae* family as the third economically important cultivated crop (Oosumi *et al.*, 2006). The family also includes raspberry and blackberry. Strawberries are of the genus *Fragaria*. There are more than 20 named species and 600 strawberry cultivars found today and they stem from five or six original wild species. The most common type of strawberries grown commercially are cultivars of the Garden strawberry (*Fragaria × ananassa*). The strawberry fruits are rich in bioactive phytochemicals, especially phenolic compounds with high antioxidant capacity, and can be beneficial to human health when they are consumed as part of the daily diet (Hannum, 2004).

Yearly strawberry production varies from 500,000 in Asian countries to 1 million tons in European countries (Gruchala *et al.*, 2004). At present 71 countries in the world are producing strawberry on 506,000 acres (Sakila *et al.*, 2007). The production of this valued fruit is mainly concentrated in North America. The United States is the largest producer of strawberries, accounting for over a quarter of total world production and the second largest total harvested area after Poland (FAOSTAT, 2007).

